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Hypochlorous acid inactivates myeloperoxidase inside phagocytosing neutrophils

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ABSTRACT

When neutrophils phagocytose bacteria, they release myeloperoxidase (MPO) into phagosomes to catalyse the conversion of superoxide to the potent antimicrobial oxidant hypochlorous acid (HOCl). Here we show that within neutrophils, MPO is inactivated by HOCl. In this study, we aimed to identify the effects of HOCl on the structure and function of MPO, and determine the enzyme's susceptibility to oxidative inactivation during phagocytosis. When hydrogen peroxide was added to a neutrophil granule extract containing chloride, MPO activity was rapidly lost in a HOCl-dependent reaction. With high concentrations of hydrogen peroxide, western blotting demonstrated that MPO was both fragmented and converted to high molecular weight aggregates. Using the purified enzyme, we showed that HOCl generated by MPO inactivated the enzyme by destroying its prosthetic heme groups and releasing iron. MPO protein was additionally modified by forming high molecular weight aggregates. Before inactivation occurred, MPO chlorinated itself to convert most of its amine groups to dichloramines. When human neutrophils phagocytosed *Staphylococcus aureus*, they released MPO that was largely inactivated in a process that required production of superoxide. Enzyme inactivation occurred inside neutrophils because it was not blocked when extracellular HOCl was scavenged with methionine. The inactivated enzyme contained a chlorinated tyrosine residue, establishing that it had reacted with HOCl. Our results demonstrate that MPO will substantially inactivate itself during phagocytosis, which may limit oxidant production inside phagosomes. Other neutrophil proteins are also likely to be inactivated. The chloramines formed on neutrophil proteins may contribute to the bactericidal milieu of the phagosome.

1. Introduction

Reactive oxidants are regarded as major components of the neutrophil's antimicrobial arsenal [1–3]. There is, however, a conundrum that must be reconciled before the role of oxidants in host defence can be fully explained. The problem is that HOCl - the chief oxidant neutrophils generate inside phagosomes - is so reactive it has a limited diffusion distance [4,5]. HOCl is likely to react with proteins close to where it is formed inside the neutrophil phagosome before it has a chance to diffuse into and kill the bacterium encased within this vacuole. Paradoxically, HOCl may limit killing of bacteria by inactivating the neutrophil's antimicrobial proteins. The protein most at risk of oxidative inactivation is myeloperoxidase (MPO) – the enzyme that generates HOCl [6]. MPO is most susceptible to inactivation because it is obviously nearest to where HOCl is formed. Along with defensins and neutrophil elastase, it is also one of the most abundant proteins in phagosomes [7]. Currently, we do not know whether MPO is subjected to oxidation during phagocytosis and, if so, how much oxidation it can tolerate before it is inactivated.

MPO is a heme enzyme that is stored within the azurophilic granules of neutrophils [6]. It is discharged into phagosomes when neutrophils ingest bacteria and fungi. Upon phagocytosis of micro-organisms, neutrophils use cytoplasmic NADPH to reduce molecular oxygen within phagosomes to superoxide [1–3]. Based on the modelling of kinetic data, most of the superoxide is predicted to react with MPO and dismutate to hydrogen peroxide [5]. MPO will then use the majority of the hydrogen peroxide to oxidize chloride to HOCl. It is now well established that

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Abbreviations:

MPO	myeloperoxidase
TMB	3,3′,5,5′-tetramethylbenzidine
DTPA	diethylenetriaminepentaacetic acid
cetrimide	alkyltrimethylammonium bromide
HBSS	Hank's Balanced Salt Solution
ELISA	enzyme-linked immunosorbent assay
FOX	ferrous oxidation in xylenol orange
Ferrozine	3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate
HOCl	hypochlorous acid
DPI	diphenylene iodonium
fMLP	*N*-formyl-methionine-leucine-phenylalanine
ABAH	4-aminobenzoic acid hydrazide
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TBST	tris-buffered saline containing Tween 20
TNB	5-thiobis-2-nitrobenzoic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)
LC-MS	liquid chromatography–mass spectrometry
PBS	phosphate buffered saline;

HOCl is generated inside neutrophil phagosomes [8–13]. From our kinetic modelling, we have estimated that up to 100 mM HOCl is produced in the first minute of phagocytosis [5]. This enormous flux of HOCl would make the phagosome an extremely oxidising environment that few bacteria would survive. The antimicrobial neutrophil proteins released into the phagosome are also exposed and potentially vulnerable to the oxidising environment.

Our kinetic model predicts that some HOCl will react with bacteria trapped inside phagosomes but only after neutrophil proteins have been substantially oxidized [5]. These predictions are supported by experimental data that show that neutrophil proteins are more heavily chlorinated than bacterial proteins [10,14]. Proteins isolated from phagosomes contained extremely high levels of 3-chlorotyrosine, a specific biomarker of HOCl [15]. Phagosomal proteins contained 12 times higher levels of 3-chlorotyrosine per tyrosine residue than the rest of the neutrophil, and six times higher levels than previously found for phagocytosed bacteria [14]. For some bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa,* sufficient HOCl reacts with them to account for killing [16,17]. In contrast, *Mycobacteria smegmatis* are not killed by the levels of HOCl formed in phagosomes [17,18].

Previous work from our laboratory suggested that elastase is unique amongst the neutrophil's antimicrobial enzymes in its ability to resist oxidative inactivation by HOCl [19]. MPO was found to be susceptible to the HOCl it generated from reagent hydrogen peroxide that was added to an extract of neutrophil granules [19,20]. Others had shown that about 40% of MPO activity in neutrophils was lost when these immune cells phagocytosed bacteria [21]. After phagocytosis, about 10% of the original activity of myeloperoxidase was found in the extracellular medium. This result indicates that some of the enzyme is released by the neutrophils during phagocytosis. The authors did not examine whether loss of activity was due to loss of MPO protein or inactivation of the enzyme. Subsequent investigations of oxygen-dependent loss of MPO activity have found that, depending on experimental conditions, superoxide, hydrogen peroxide or HOCl can inactivate the enzyme [22–25].

HOCl reacts rapidly with the heme prosthetic groups of the enzyme, converting them to the redox intermediate Compound I [26,27]. The rate constant for this reaction is about $2 \times 10^8\ M^{-1}\ s^{-1}$, which is 10-fold faster than the reaction of hydrogen peroxide with the native enzyme [26]. Consequently, HOCl can be expected to react with MPO in physiological situations, especially when other targets for the oxidant have been exhausted. In the absence of scavengers, HOCl inactivates the enzyme, oxidizes its amino acid residues, and promotes destruction of the heme prosthetic groups with resulting loss of iron [24,27]. Cysteine and methionine residues will be most susceptible to oxidation because they are preferentially oxidized by HOCl [28].

In this investigation we aimed to establish how much oxidation MPO can tolerate before it is inactivated. We also directly addressed the question of whether MPO promotes its own oxidative inactivation during phagocytosis of bacteria.

2. Experimental procedures

2.1. Reagents

Myeloperoxidase (MPO), purified from pooled human blood, was supplied by Planta Natural Products (Vienna, Austria). Its purity index (A_{430}/A_{280}) was at least 0.80 and its concentration was determined per heme group ($\varepsilon_{430} = 91{,}000\ M^{-1}\ cm^{-1}$) [29]. Stock hypochlorous acid was household chlorine bleach, (Janola, Pental Ltd, Australia), from which dilutions were made daily ($\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$ at pH 12) [30]. Hydrogen peroxide (30% solution, LabServ Pronalys) was also diluted freshly and its concentration determined for each experiment ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$) [31]. All reagents including 3,3′,5,5′-tetramethylbenzidine (TMB), diethylenetriaminepentaacetic acid (DTPA), alkyltrimethylammonium bromide (cetrimide), superoxide dismutase (from bovine erythrocytes), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (Ferrozine), L-ascorbate, diphenylene iodonium (DPI), cytochalasin B and *N*-formyl-Met-Leu-Phe (fMLP) were from Sigma, unless otherwise stated. Dimethylformamide (DMF) was from J. T. Baker™. Dextran (200–300 kDa) was from MP Biomedicals. Ficoll-Paque™ Plus and Amersham™ ECL Plus Western Blotting Detection System were from Cytiva.

2.2. Isolation of neutrophils

Blood was collected from healthy human volunteers with informed consent, and with ethical approval from the Southern Health & Disability Ethics Committee, New Zealand. Neutrophils were isolated under sterile conditions from freshly drawn heparinized blood as previously described [32]. Briefly, dextran sedimentation was followed by Ficoll-Paque centrifugation, and erythrocyte lysis in hypotonic buffer. Neutrophils were >96% pure as determined by flow cytometry using their characteristic forward/side scatter, and were counted using a haemocytometer.

2.3. Preparation of neutrophil granule extracts

Neutrophils, 10^7/mL in Hank's balanced salt solution (HBSS) were incubated with 10 μM DPI and 5 μg/mL cytochalasin B for 10 min at 37 °C. Degranulation was stimulated by the addition of 1 μM fMLP, after which cells were incubated for 20 min at 37 °C and gently mixed every 5 min. The neutrophils were pelleted (5 min, 500 g at 4 °C) and the supernatant was collected as the neutrophil granule extract. For hydrogen peroxide treatment, the neutrophil granule extract was warmed to 37 °C and hydrogen peroxide was added in small volumes while vortexing. Each dose provided 250 μM hydrogen peroxide, and was added at 5 min intervals until the total dose was achieved. Where indicated, 5 mM or 10 mM methionine or 20 μM of the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH) was present before hydrogen peroxide was added [33]. MPO activity in the granule extract was determined before and after treatments using either the MPO ELISA or TMB oxidation assay.

2.4. Measurement of MPO activity and protein by ELISA

The MPO ELISA allows consecutive activity and protein

measurements after capturing MPO with a monoclonal antibody. In brief, activity was measured by oxidation of Amplex™ UltraRed (Invitrogen), then MPO protein was quantified using a polyclonal antibody against MPO followed by a biotin-avidin-alkaline phosphatase detection system. A detailed protocol is provided in the Supplementary Material. The specific activity of MPO was calculated as the ratio of MPO activity to MPO protein.

2.5. TMB assay for MPO activity

In this assay, oxidation of TMB to a blue product reports on the total utilisation of hydrogen peroxide by MPO encompassing peroxidation and halogenation activities depending on the conditions [34]. The standard conditions for testing aliquots of either neutrophil granule extract or purified MPO were 200 mM sodium acetate buffer pH 5.4 containing 0.01% cetrimide, 2 mM TMB and 200 μM hydrogen peroxide. The stock solution of TMB (20 mM in DMF) was prepared freshly, and was combined with hydrogen peroxide just prior to use to avoid auto-oxidation. To measure the residual activity of purified MPO after oxidant treatment, an aliquot from the treatment incubation was diluted 40-fold into a cuvette containing the acetate buffer mixture and TMB oxidation by MPO (0.8 nM) was monitored by absorbance at 670 nm at room temperature.

2.6. Conversion of hydrogen peroxide to HOCl by MPO in the presence of chloride

The conversion of hydrogen peroxide to HOCl by 1.5 μM MPO in the presence of 140 mM NaCl in 50 mM sodium phosphate buffer pH 7.4 containing 100 μM DTPA and 5 mM taurine was determined for a concentration range of 10 μM–750 μM of hydrogen peroxide using the taurine chloramine assay.

2.7. Taurine chloramine assay to measure HOCl production

Taurine reacts rapidly with HOCl as it is generated, to form the stable product taurine chloramine, which was then measured within an hour by the iodide catalyzed oxidation of TMB as previously described [35]. To assess MPO's conversion of different concentrations of hydrogen peroxide to HOCl, 5 mM taurine was included in the reaction buffer. In a 96-well plate, 80 μL of sample was added to 20 μL of developing solution (400 mM sodium acetate buffer pH 5.4, 2 mM TMB, 100 μM sodium iodide), then after 13 min the absorbance at 670 nm was measured and calculated against a taurine chloramine standard curve.

2.8. FOX assay for measurement of hydrogen peroxide

Ferrous oxidation with xylenol orange was used to measure residual hydrogen peroxide concentrations [36]. The FOX reagent was composed of 1 mM ammonium ferrous sulfate, 400 μM xylenol orange and 400 mM D-sorbitol in 200 mM sulfuric acid. Each peroxide assay was performed by adding 70 μL of sample to 25 μL FOX reagent while vortexing, followed by an incubation of 45 min at room temperature in the dark prior to reading the absorbance at 560 nm. The hydrogen peroxide concentration was calculated against a standard curve of the range of 0–5 nM hydrogen peroxide. Samples with concentrations higher than the standard curve range were diluted accordingly in 50 mM sodium phosphate buffer pH 7.4 before adding to the FOX reagent.

2.9. SDS-PAGE and western blotting analyses

2.9.1. SDS-PAGE analysis and western blotting of neutrophil granule extract

Aliquots of hydrogen peroxide treated granule extract were resolved using 12% SDS-PAGE under reducing conditions, followed by silver staining. For western blot analysis PVDF membranes were blocked for 1 h in 5% skim milk in TBST (50 mM Tris-HCl buffer pH 7.4 with 140 mM NaCl containing 0.05% Tween). All antibodies were diluted in 2% skim milk in TBST, and primary antibody incubations were over night at 4 °C at 1:1000 for MPO (rabbit polyclonal antiserum produced in-house), and at 1:10,000 for lactoferrin (polyclonal rabbit antibody, DAKO A186). Following a 1 h incubation with secondary antibody (1:10,000 dilution of goat anti rabbit peroxidase conjugate), blots were developed using enhanced chemiluminescence (ECL) and analysed on a ChemiDoc® XRS (Bio-Rad). For Fig. 1F granule extract aliquots were precipitated using 20% (v/v) trichloroacetic acid, 20% (v/v) acetone and 0.1% (w/v) sodium deoxycholate. Air dried precipitates were resuspended in 20 μL reducing SDS-PAGE loading buffer (2% (w/v) SDS, 10% (v/v) glycerol, 125 mM Tris-HCl buffer pH 6.5, 1% (v/v) β-mercaptoethanol). Samples (8 μL) were loaded without prior heating onto 8–20% gradient SDS-PAGE and blotted as above.

2.9.2. SDS-PAGE analysis of myeloperoxidase

To activate the generation of HOCl, MPO (1.5 μM) was incubated in 50 mM sodium phosphate buffer pH 7.4 containing 140 mM NaCl at room temperature with the following hydrogen peroxide to protein ratios: 10:1, 25:1, 50:1, 100:1, 200:1, 300:1, 400:1, 500:1, 1000:1 and 1500:1. Hydrogen peroxide concentrations above 300 μM were added in several aliquots of up to 300 μM with 10 min between additions. After 1 h total incubation time, 15 μL aliquots were added to non-reducing and reducing sample loading buffer. Samples were loaded without prior heating onto 8–20% gradient gels, stained with Coomassie Brilliant Blue R-250 for 60 min followed by destaining, and scanned using ChemiDoc® XRS (Bio-Rad). For treatment with reagent HOCl, MPO (1.5 μM) was suspended in 50 mM sodium phosphate buffer pH 7.4 containing 100 μM DTPA. Aliquots (15 μL) were run on gradient SDS-PAGE and stained as described above.

To detect intact covalently-bound heme of MPO before and after the treatments described above, samples were resolved by SDS-PAGE and blotted onto a PVDF membrane (100 V, 1 h). The membrane was subsequently probed for *in situ* heme activity using chemiluminescence as described previously [37].

2.10. Spectral analysis of MPO

Time resolved spectra of 1.5 μM MPO in 50 mM sodium phosphate buffer pH 7.4 containing 140 mM sodium chloride and 100 μM DTPA were recorded (Agilent 8453 diode array spectrophotometer) until no further absorbance change was observed. The reaction was started by the addition of hydrogen peroxide in varying hydrogen peroxide to protein ratios (5:1, 10:1, 50:1, 100:1 and 200:1). HOCl generation by the MPO/H_2O_2/Cl^- system was determined by the taurine chloramine assay as described above. Absence of residual hydrogen peroxide was tested by the ferrous oxidation-xylenol orange (FOX) assay. 40 μg/mL SOD was added to a separate set of reactions at the highest oxidant to protein ratio.

Spectra were also recorded after HOCl was added to MPO at concentrations as for hydrogen peroxide but without sodium chloride in the buffer. For both oxidant systems, spectra were taken after 1 h to determine the residual absorbance of the heme Soret peak at 430 nm. At 1 h, all generated or added HOCl had reacted as checked by the taurine chloramine assay. 100 μM of ascorbate was added to check if all MPO had converted back to the ferric form after 1 h.

2.11. Analysis of free iron

Release of free iron from the heme prosthetic group was measured colorimetrically using ferrozine following a published method with slight modifications [38]. After buffer exchange to 50 mM sodium acetate buffer pH 7.4 using a Micro Bio-Spin chromatography column (Biorad), MPO (1.5 μM) was treated either with 2.25 mM hydrogen peroxide (300 μM aliquots added in 7.5 doses at 10 min intervals) in the

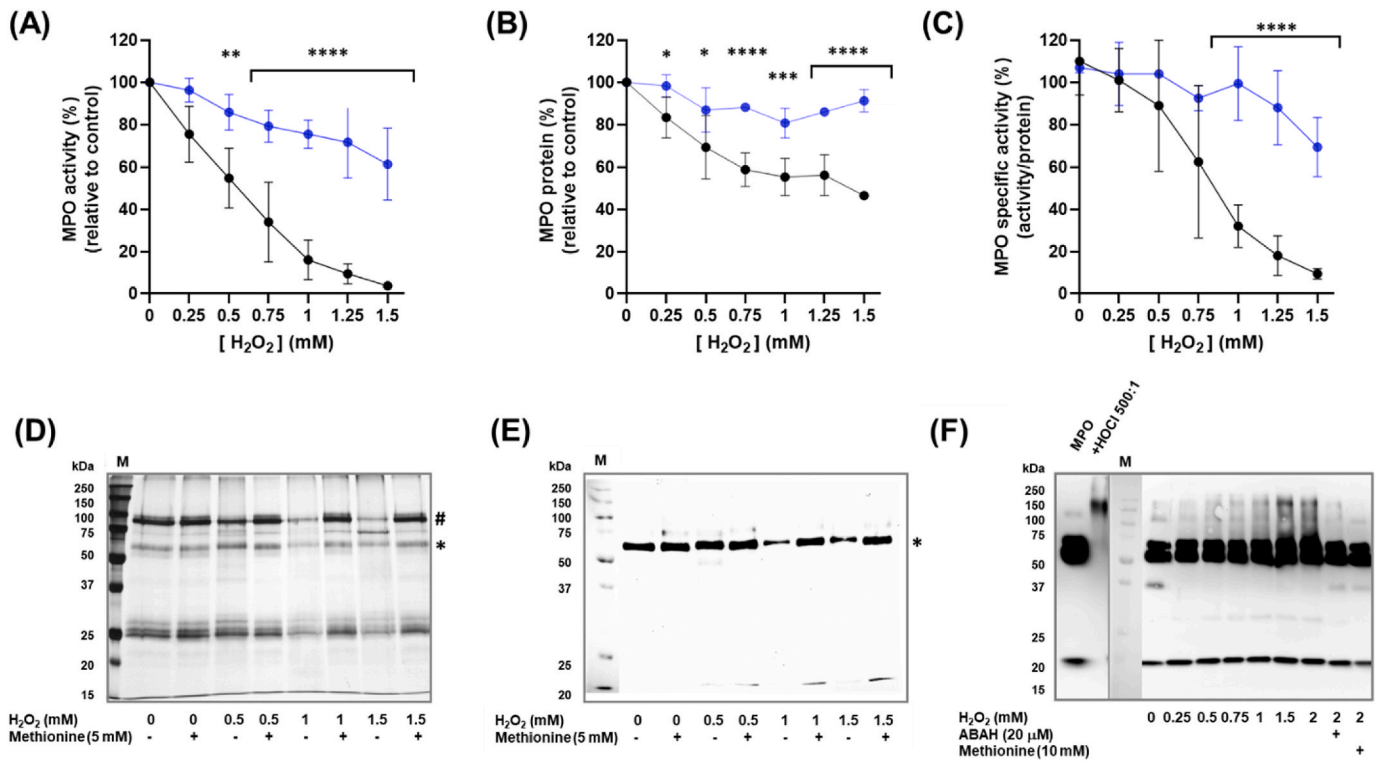

Fig. 1. Inactivation of MPO in a neutrophil granule extract after the addition of hydrogen peroxide in the absence and presence of methionine. Neutrophil granule extracts were treated with consecutive doses of 250 µM hydrogen peroxide, with 5 min incubation between doses at 37 °C in the absence (●) and presence of 5 mM methionine (•), and samples were taken after each addition of hydrogen peroxide and analysed immediately by ELISA for **(A)** activity (n = 4) and **(B)** protein levels of MPO (n = 4). **(C)** Specific activity, the ratio of MPO activity to MPO protein, was calculated using paired activity and protein results of four independent experiments. All points are mean ± SD and significant differences ± methionine were determined by two-way ANOVA analysis followed by Šidák's multiple comparisons test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). **(D)** Silver staining and **(E)** western blot analysis of MPO of neutrophil granule extracts were prepared and treated above and subjected to 12% SDS PAGE under reducing conditions. MPO is marked with * and lactoferrin with # (see Fig. S2). **(F)** Western blot analysis of MPO from neutrophil granule extracts that were precipitated after oxidant treatment and resolved under reducing conditions on a 4–20% SDS-PAGE. Images are representative of four independent experiments.

presence of 140 mM chloride, or 750 µM reagent HOCl and incubated at room temperature for 2 h. The reaction volume of 1 mL was dried by speed-vac. The pellet was resuspended in 30 µL water to which 30 µL of ascorbate solution (1.13 mM in 0.2 M HCl) was added and left for 5 min. Protein was removed by precipitation by adding 30 µL of 11.3% trichloroacetic acid, keeping on ice for 5 min before spinning briefly at 4 °C. The iron content of the supernatant was determined by adding 36 µL of 10% ammonium acetate followed by 9 µL of 6.1 mM ferrozine, and reading the absorbance at 563 nm once colour development was complete (5 min for MPO reacted with HOCl, 2 h for MPO reacted with hydrogen peroxide and chloride).

2.12. Measurement of chloramines on oxidatively modified MPO

Chloramines react with the yellow 5-thiobis-2-nitrobenzoic acid (TNB) (ε = 13,100 $M^{-1}cm^{-1}$ at 412 nm) to generate colourless 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [39]. A TNB stock solution was prepared by adding NaOH to a 2 mM stock solution of DTNB to raise the pH to 12. The pH was kept at 12 for 5 min before HCl was added to lower the pH to 7.4. The solution was prepared freshly and kept on ice in the dark. A standard curve in the range of 5–40 µM HOCl was generated by reacting HOCl with 5 mM taurine in 50 mM sodium phosphate buffer pH 7.4.

MPO (0.25 µM) was reacted with hydrogen peroxide in the presence of sodium chloride or HOCl, in 50 mM sodium phosphate buffer pH 7.4 at various oxidant to protein ratios. 10, 25, 50, 75, 100 and 125 µM of the respective oxidant was added, which equates to an oxidant per amine ratio of 0.5, 1.1, 2.3, 3.4, 4.6 and 5.7, when taking a total of 22 amine groups into account (comprised of 14 lysine, 6 histidine and 2 α-amino groups). After 10 min at room temperature, aliquots were added to the TNB solution and loss of absorbance at 412 nm was related to chloramine concentration using the taurine chloramine standard curve. No HOCl was left after 10 min due to its high reactivity with other amino acid residues on MPO (23 methionine and 17 cysteine residues next to a number of other amino acid residues which react with HOCl, e.g. tryptophan and tyrosine residues [28]). Chloramine formation was expressed as mono- or dichloramines formed per available amine group of one MPO monomer consisting of a heavy and light chain (a total of 22 amine groups as stated above).

2.13. MPO polymer analysis by size exclusion HPLC

MPO (1.5 µM) in 50 mM sodium phosphate buffer pH 7.4 containing 140 mM NaCl was treated with the following hydrogen peroxide to protein ratios: 400:1, 500:1, 1000:1 and 1500:1 for 1 h at room temperature. Hydrogen peroxide was added in 300 µM aliquots with 10 min between additions. MPO (1.5 µM) was also reacted with reagent HOCl in 50 mM sodium phosphate buffer pH 7.4. Oxidant to protein ratios were 50:1, 250:1, 400:1 and 500:1. Samples were centrifuged (20,000 g for 5 min) to remove any particulate prior to injection (56 µL) onto a Biosep-SEC S3000 column (Phenomenex) on a Waters 2690 HPLC instrument. Separation was achieved with a flow-rate of 0.5 mL/min of 50 mM sodium phosphate buffer pH 7.4 containing 0.5 M NaCl, with online detection at 280 and 430 nm. Elution was calibrated using protein standards of ferritin (440 kDa), catalase (240 kDa), MPO (146 kDa), bovine serum albumin (67 kDa) and ribonuclease (14 kDa), with elution

times of 15.1, 16.5, 17.2, 17.5 and 20.2 min, respectively.

2.14. Dynamic light scattering to determine myeloperoxidase polymer size

DLS is a widely used technique for measuring the size of proteins, polymers and other molecules in solution. The translational diffusion coefficient of particle motion is determined by measuring the dynamic fluctuations in light scattering intensity caused by the Brownian motion of the particle. This method provides a hydrodynamic radius that can be calculated using the Stokes-Einstein equation [40]. Using the same oxidant to protein ratios as for size exclusion HPLC, 5 μM MPO in 50 mM sodium phosphate buffer pH 7.4 containing 467 mM NaCl was treated with the following hydrogen peroxide to protein ratios: 400:1, 500:1 and 1000:1. Reagent HOCl was added to MPO (5 μM) at the ratios of 50:1, 100:1 and 200:1. Samples were incubated for 1 h at room temperature before analysis on a DynaPro NanoStar (Wyatt) light scattering detector to determine the particle size distribution and weight-average molar mass of polymers.

2.15. Electronic circular dichroism

MPO (5 μM) was treated with reagent HOCl at the ratio of 200:1 in 50 mM sodium phosphate buffer pH 7.4 and compared to untreated MPO. Electronic circular dichroisms (ECD) spectra were monitored as a function of temperature using Chirascan (Applied Photophysics, Leatherhead, UK). The instrument was equipped with a Peltier element for temperature control. Single wavelength scans were performed in the far-UV with following instrumental parameters: pathlength: 1 mm; spectral bandwidth: 1 mm, scan time per point: 12 s; The thermal denaturation was followed at 222 nm, heating from 20 °C to 104 °C with a heat rate of 1 °C/min. The fraction α of unfolded protein was calculated according to $\alpha = (\theta N - \theta T)/(\theta N - \theta U)$ with θN being the ellipticity at 222 nm of the protein in the native folded state, θT the ellipticity at defined temperature (T), and θU being the ellipticity at 222 nm of the completely unfolded state. From the fraction of unfolded protein an equilibrium constant *K*eq for each temperature can be calculated with following equation: $Keq = \alpha/(1-\alpha)$. By plotting *R*ln*K*eq against the inverse of temperature, the thermodynamic parameters could be calculated [41].

2.16. Neutrophil phagocytosis experiments

For the standard procedure, phagocytosis was carried out in 400 μL volumes in 2 mL plastic tubes (Tarsons) containing neutrophils (10^7/mL) and serum-opsonised *Staphylococcus aureus* (2×10^8/mL) in HBSS with 10% serum with end-over-end mixing (6 rpm) at 37 °C. The bacteria (*S. aureus* strain 502a, ATCC 27217) were prepared as follows: after harvesting by centrifugation from an overnight culture in Lysogeny broth, bacteria were washed twice by centrifugation in PBS, and resuspended in HBSS. Using optical density to estimate their concentration (for 502a an OD_{550} 0.36 ≈ 4×10^8/mL), bacteria were opsonised just prior to use by incubating them (4×10^8/mL) for 20 min at 37 °C with end-over-end rotation in HBSS containing 10% serum (pooled from at least six healthy donors). For the inhibitor DPI, neutrophils (2×10^7/mL) were preincubated for 10 min at 37 °C with 10 μM DPI before the addition of an equal volume of opsonised bacteria to start the phagocytosis incubation. In the case of methionine, it was added to neutrophils just prior to the addition of bacteria, for a final concentration of 1 mM. After the 30 min phagocytosis incubation, neutrophils were gently pelleted by centrifugation at 100*g* in a swing-out rotor, for 5 min at 4 °C. The supernatant was collected, as the extracellular fraction containing released MPO, and was diluted between 1:10 and 1:80 when applied to the MPO ELISA. See Supplementary Material for details of the preparation of released MPO samples for LC-MS analysis, and for the neutrophil experiments generating samples for CM-sepharose extraction prior to measuring peroxidase activity with the TMB assay.

LC/MS/MS/Orbitrap: *Analysis of MPO residues oxidized by H_2O_2/Cl^- treatment.*

A method was developed to determine which amino acid residues of MPO are modified by self-oxidation in the MPO/H_2O_2/Cl^- system. LC-MS/MS of tryptic peptides was performed on a highly sensitive Ultimate 3000 uHPLC (Thermo Scientific) coupled to LTQ Orbitrap XL system (Thermo Scientific) at the Centre for Protein Research, University of Otago, Dunedin. Each sample was analysed 4 times using the same instrument settings. See Supplementary Material for details of the method, instrumentation and analysis.

LC/MS/MS/QTrap: *Identification of 3-chlorotyrosine (Cl-Tyr) on neutrophil-extracted MPO.*

This method was developed to determine whether MPO was oxidized in activated neutrophils. A multiple reaction monitoring (MRM) method was set up after identifying target Cl-Tyr peptides using purified MPO in an H_2O_2/Cl^- oxidising system (Table S1). This preliminary work was done using a Dionex UltiMate 3000 HPLC (Thermo Scientific) coupled to a Velos Pro ion trap MS (Thermo Scientific). Then samples from neutrophil experiments were analysed by MRM using an Infinity 1290 LC (Agilent) coupled to 6500 QTrap MS (Sciex). For details see Supplementary Material.

3. Results

3.1. Self-inactivation of myeloperoxidase in neutrophil granule extracts

We first confirmed earlier results showing that MPO inactivates itself with hypochlorous acid (HOCl) when hydrogen peroxide is added to a neutrophil granule extract [19,20]. To gain greater insight into how MPO is modified when it is present with other neutrophil proteins, we used an ELISA to concurrently measure MPO activity and MPO protein. Addition of hydrogen peroxide to the neutrophil granule extract containing 140 mM NaCl caused a loss of MPO activity that was dependent on the hydrogen peroxide concentration (Fig. 1A). Methionine, which scavenges HOCl but not hydrogen peroxide, largely attenuated the loss of enzyme activity. This result confirms that the loss of enzyme activity was caused predominantly by HOCl generated by MPO. The same loss of activity was measured using an alternative non-ELISA assay (Fig. S1A). Detection of MPO protein by ELISA also declined as increasing concentrations of hydrogen peroxide were added to the extract (Fig. 1B). The decline in MPO protein levels was attenuated by methionine. This result suggests that MPO protein was modified by HOCl such that less of the protein was either bound by the capture antibody or recognised by the detecting antibody. At high doses of hydrogen peroxide, only 50% of the MPO protein was detected by the ELISA. Interestingly, peroxidase activity measured by the ELISA and the TMB assay matched remarkedly well (Fig. S1A). This result suggests that the MPO not detected by the ELISA was largely inactive. Tracking the specific activity of MPO (Fig. 1C), which is the ratio of enzyme activity to MPO protein, was informative because it showed that at low concentrations of hydrogen peroxide the decrease in measured activity (Fig. 1A) was explained mainly by the drop in measured protein (Fig. 1B) due to modification of the protein so that it was not recognised in the ELISA. However, at higher concentrations of hydrogen peroxide, where the drop of detected protein had plateaued, the continual loss of enzyme activity is best explained by modifications of MPO that are directly related to its activity.

We used gel electrophoresis and western blotting to assess alterations to MPO protein after treatment of the granule extract with hydrogen peroxide (Fig. 1D–F). Silver staining (Fig. 1D) and western blotting (Fig. 1E) showed that treating the granule extract with increasing concentrations of hydrogen peroxide in the presence of chloride led to decreased MPO band intensities, which was blocked by methionine. Other proteins in the extract were affected when MPO generated HOCl. The most noticeable change was the loss of a 90 kDa protein (Fig. 1D), which was identified as lactoferrin (Fig. S1B). Concentration of the samples by protein precipitation prior to SDS-PAGE revealed that some

MPO formed non-reducible aggregates of high molecular weight structures (Fig. 1F). The MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH) or methionine decreased formation of the aggregates. These results showed that protein aggregation required MPO activity and production of HOCl and that hydrogen peroxide alone was not responsible for the changes to granule proteins observed in these experiments.

3.2. Self-inactivation of purified myeloperoxidase

To get a more precise estimate of the susceptibility of MPO to self-inactivation than could be achieved with the granule extract, we investigated how purified MPO lost activity when it generated HOCl. The residual activity of MPO was measured after it had reacted with hydrogen peroxide in the presence of chloride (Fig. 2A) or reagent HOCl (Fig. 2B). MPO did not lose substantial activity until a ratio of at least 200 mol hydrogen peroxide was added to 1 mol of enzyme (Fig. 2A). At a molar ratio of 500:1, about 40% of the enzyme activity was lost. In previous work we found that much higher concentrations of hydrogen peroxide were required to inactivate MPO when chloride was not present in the buffer, which confirms that HOCl not hydrogen peroxide was responsible for the observed enzyme inactivation [23].

In contrast to self-generated HOCl, MPO was highly susceptible to reagent HOCl (Fig. 2B). At a molar ratio of only 50:1, half of the enzyme was inactivated. Interestingly, physiological concentration of chloride attenuated enzyme inactivation even at high doses of HOCl. When MPO was treated with HOCl at a 50:1 oxidant to protein ratio, inactivation occurred rapidly within the first 2 min with no further loss of activity after 10 min (Fig. 2C). These results demonstrate that purified MPO is rapidly inactivated by its product HOCl. Inactivation, however, can be inhibited with chloride, which turns over Compound I and restores ferric MPO. These data suggest that the mechanism of inactivation involves the initial formation of Compound I, the redox intermediate of MPO that reacts with chloride.

To determine whether HOCl reacts with the active site of MPO and alters its redox state, time resolved spectra of the reaction of MPO were followed when the enzyme reacted with hydrogen peroxide in the presence of chloride. The heme prosthetic groups in the native enzyme have a Soret maximum at 430 nm and a smaller visible peak at 570 nm (Fig. 2D). Adding hydrogen converted MPO to its Compound II redox intermediate within seconds, as seen by the shift of the Soret peak to 452 nm and the increase in absorbance at 630 nm. Higher concentrations of hydrogen peroxide caused more complete conversion of the enzyme to Compound II (not shown). The initial conversion to Compound II was followed by a slower decay back to the native enzyme within 2 min and a concomitant loss of more than 50% of the heme Soret peak. Recovery of the native enzyme decreased with increasing

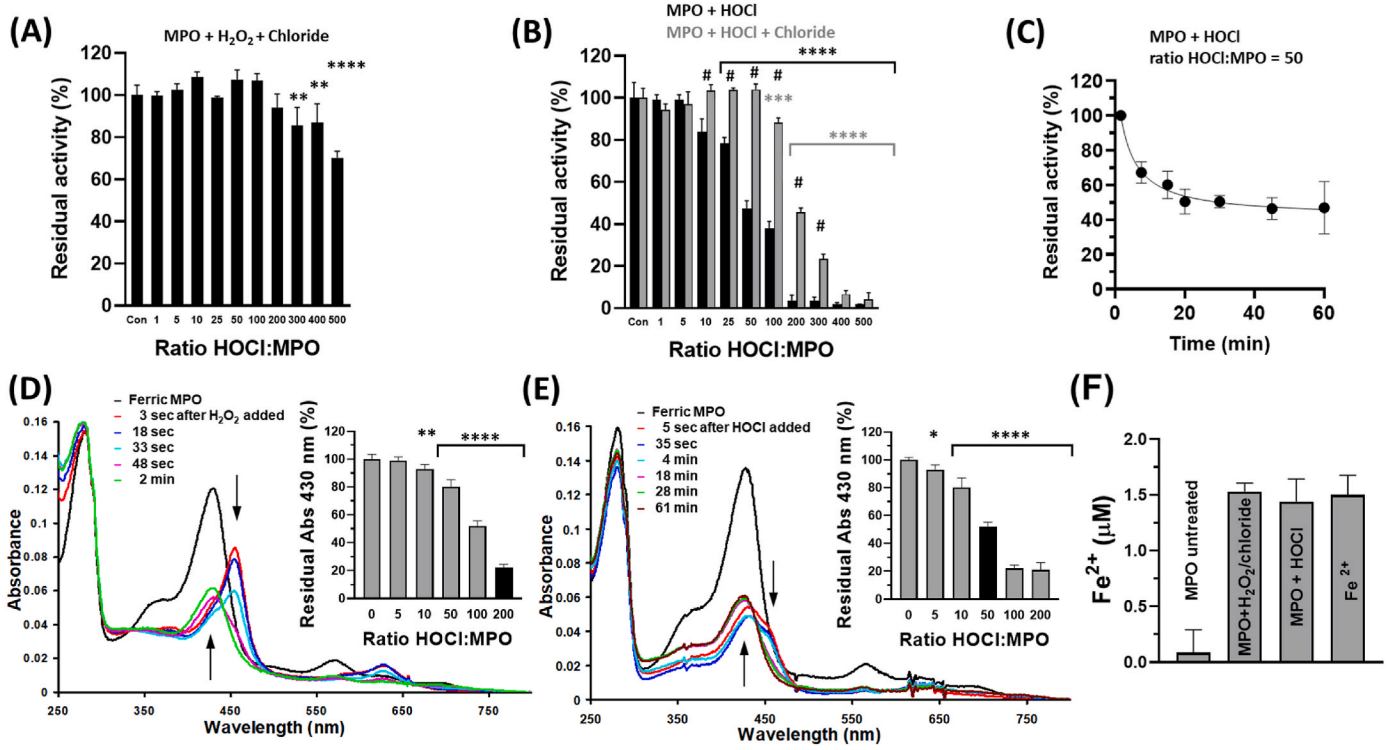

Fig. 2. Inactivation of purified MPO. (A) Loss of peroxidase activity after treating 32 nM of MPO with hydrogen peroxide in the presence of 140 mM NaCl in 50 mM phosphate buffer pH 7.4 containing 100 μM DTPA. Residual peroxidase activity using the TMB assay was determined by taking aliquots after 60 min of incubation at room temperature. Differences to untreated control were determined by one-way ANOVA followed by Dunnett's multiple comparisons test (**$p < 0.01$; ****$p < 0.0001$). **(B)** Loss of peroxidase activity after treating 32 nM MPO with HOCl in the absence (black) and presence of 140 mM NaCl (grey) under conditions described above. ***$p < 0.001$; ****$p < 0.0001$ by one-way ANOVA as in (A), black * for HOCl without and grey * for with chloride. # depict differences between ± chloride by two-way ANOVA fitting a mixed model. **(C)** Time course for loss of peroxidase activity after MPO (32 nM) was treated with 50-fold molar excess of reagent HOCl under conditions described above. **(D)** Spectral changes of MPO were monitored after treating 1.5 μM MPO with 300 μM H_2O_2 (molar ratio of 200, shown in black in the insert) in the presence of 140 mM NaCl in 50 mM phosphate buffer pH 7.4. Black arrows indicate direction of spectral changes. Inset: % Residual absorbance of the heme Soret peak at 430 nm was measured 45 min after 1.5 μM MPO was reacted with hydrogen peroxide in the presence of 140 mM NaCl at the specified oxidant to protein ratios. **(E)** Spectral changes of MPO were monitored after treating MPO with 75 μM HOCl (molar ratio of 50, shown in black in the inset) under the conditions described above. Inset: % Residual absorbance of the heme Soret peak at 430 nm was measured 1 h after 1.5 μM MPO was reacted with reagent HOCl at the oxidant to protein ratios indicated. Insets of (D) and (E): *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ by one way ANOVA and Dunnett's multiple comparion. **(F)** Release of iron from 1.5 μM MPO after treating it with 7.5 aliquots of 300 μM hydrogen peroxide in the presence of 140 mM chloride or with 750 μM reagent HOCl in 50 mM phosphate buffer. 1.5 μM Fe^{2+} was used as a positive control. Fe^{2+} was determined colorimetrically with ferrozine. All plots represent the mean of $n \geq 3 \pm$ SD and spectra are representative of 3 independent experiments.

concentrations of hydrogen peroxide (Fig. 2D inset). Compound II is formed by reduction of compound I by hydrogen peroxide, with production of superoxide [42]. Addition of superoxide dismutase, however, had no effect on the extent of compound II formation or the recovery of native enzyme (not shown). HOCl generation by the MPO/H_2O_2/Cl^- system was confirmed by the taurine chloramine assay. Up to a concentration of 300 μM, hydrogen peroxide was converted entirely to HOCl. Higher concentrations of hydrogen peroxide were predominantly converted to HOCl (450 μM, 600 μM and 750 μM of hydrogen peroxide were converted to 400 ± 21 μM, 470 ± 42 μM and 530 ± 14 μM, respectively; n = 3, SD). All hydrogen peroxide was consumed when no more spectral changes were detectable as tested by the ferrous oxidation-xylenol orange (FOX) assay (data not shown).

We next followed the spectral changes of MPO after it had reacted with reagent HOCl in the absence of chloride (Fig. 2E). Upon addition of the oxidant there were rapid decreases in absorbance at 430 nm and 570 nm with the appearance of a shoulder at 456 nm. The spectral species observed slowly decayed back to ferric MPO and no more spectral changes were observed after 60 min. Recovery of the native enzyme declined with increasing concentrations of HOCl (Fig. 2E Inset). Addition of ascorbate, which reacts rapidly with Compound I and II of MPO, did not restore more of the heme Soret peak in both systems, confirming that the spectral changes observed were not due to incomplete conversion of redox intermediates. No spectral changes were detected at 280 nm, which confirms that the loss of heme Soret peak was not due to loss of protein e.g. due to precipitation (Fig. 2D and E). These results demonstrate that HOCl generated by MPO reacts rapidly with the heme prosthetic groups, leading to their eventual destruction. To confirm that the heme groups of MPO had been destroyed, we measured the release of iron from MPO after it had been heavily oxidized. Treatment of 1.5 μM MPO with 7.5 additions of 300 μM hydrogen peroxide in the presence of chloride resulted in the total release of heme iron (Fig. 2F). Reagent HOCl liberated approximately 95% of iron from the heme prosthetic groups.

To assess whether HOCl reacted with amino acid residues on MPO before the enzyme was inactivated, we measured chloramine formation on the protein after it had been treated with hydrogen peroxide in the presence of chloride, or with reagent HOCl (Fig. 3A). The extent of chloramine formation on the protein was related to its content of available lysine and histidine residues, as well as the α-amino groups of the light and heavy chains of one MPO monomer. Chloramines were formed on the protein by both oxidant treatments. Chloramines, shown as the amount formed per available amine group, increased as ratio of oxidant to amine group was increased (Fig. 3A). At low doses, most of the oxidant was accounted for by monochloramines formed on MPO. As the dosage of oxidant was increased, close to the maximum amount of dichloramines were formed on MPO. These results demonstrate that when HOCl reacts with MPO, amine residues will be major targets and converted to chloramines and dichloramines.

Oxidative changes to purified MPO when it reacted with hydrogen peroxide in the presence of chloride (Fig. 3B), or with reagent HOCl (Fig. S2A), were also analysed by gel electrophoresis. Under non-

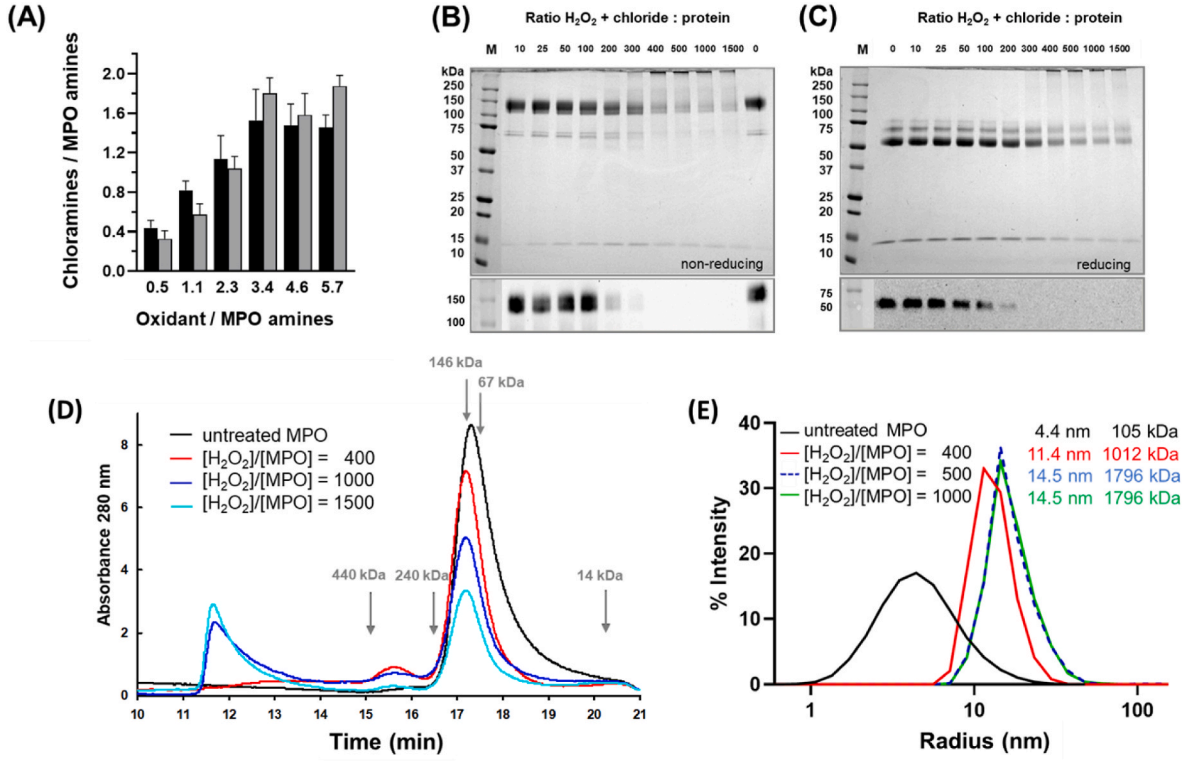

Fig. 3. Modifications to MPO protein after oxidant treatment: (A) Protein chloramines were measured with TNB after treating 0.25 μM MPO with hydrogen peroxide in the presence of 140 mM NaCl (black bars), or with reagent HOCl (grey bars) in 50 mM phosphate buffer pH 7.4 at the indicated ratio of oxidant to the number of amines on MPO (see Methods). Mean of n ≥ 3 ± SD. **(B and C)** SDS PAGE Coomassie R250 stain of MPO reacted with increasing amounts of hydrogen peroxide in the presence of chloride. Top panels: 1.5 μM MPO was treated with hydrogen peroxide in 50 mM phosphate buffer pH 7.4 and 140 mM NaCl, then resolved on an 8–20% gradient gel under non-reducing (B) or reducing conditions (C). Untreated MPO is labelled as Con. Bottom panels: The SDS-PAGE gels were blotted onto PVDF membrane and MPO heme was detected by chemiluminescence. **(D)** Size exclusion chromatography of 1.5 μM MPO treated with hydrogen peroxide in the presence of 140 mM chloride in 50 mM phosphate buffer pH 7.4. The hydrogen peroxide to protein ratios were 400:1 (red), 1000:1 (blue) and 1500:1 (turquoise). Untreated control is shown in black. Molecular weight standards used to estimate the size of the polymers are shown in grey and their elution times are indicated by the grey arrows. Hydrogen peroxide was added in 300 μM aliquots. **(E)** Dynamic light scattering of 5 μM MPO treated with hydrogen peroxide in 50 mM sodium phosphate buffer pH 7.4 containing 467 mM NaCl. Hydrodynamic radii and average molecular weight of each condition are stated on the graph in the corresponding colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reducing and unheated conditions, untreated MPO ran chiefly as a single diffuse band at the expected molecular weight of MPO (146 kDa, Fig. 3B and Fig. S2A, lane labelled 0). MPO protein consists of two disulfide-linked heterodimers which are comprised of a glycosylated heavy subunit (58.5 kDa) and one light subunit (14.5 kDa) covalently linked via the heme prosthetic group. On some gels a faint band of 14.5 kDa was also visible for untreated MPO, indicating that a minor fraction of the light chain can detach from the homodimer under non-reducing SDS PAGE conditions (Fig. 3B and Fig. S2A). Under reducing SDS-PAGE conditions, untreated MPO should theoretically appear as a single polypeptide band with a molecular weight of 73 kDa for the monomer consisting of one heavy and one light subunit. However, we observed two additional bands with molecular weights of approximately 60 kDa and 14 kDa (Fig. 3C and Fig. S2B), which are likely to be the heavy and light chains respectively.

When MPO was treated with oxidant and resolved on SDS PAGE under non-reducing (Fig. 3B) or reducing conditions (Fig. 3C), all bands decreased in intensity as the dose of oxidant increased. The single diffuse band of untreated MPO under non-reducing conditions (Fig. 3B) became a distinct doublet with bands of approx. 130 kDa and approx. 115 kDa. These bands are consistent with the respective loss of one and two light subunits from the full protein. Additionally, non-reducible high molecular weight polymers were observed from an oxidant to protein ratio of 300:1, and became the dominant band at higher doses. The reaction of MPO with reagent HOCl also showed a similar development of non-reducible high molecular weight polymers (Figs. S2A and S2B). Polymer formation with reagent HOCl occurred rapidly at a 500:1 oxidant to protein ratio and was nearly completed after 5 min (data not shown).

Identical gels as described above were blotted onto a PVDF membrane and enhanced chemiluminescence was used to detect the heme prosthetic group. The strong signal of MPO at 146 kDa under non-reducing conditions (Fig. 3B and S2A, bottom panels) and at 73 kDa for reducing conditions (Fig. 3C and S2B, bottom panels) progressively decreased with increasing HOCl generated via the MPO/H_2O_2/Cl^- system (Fig. 3B and C, bottom panels) or reagent HOCl added (Figs. S2A and S2B, bottom panels). No additional chemiluminescence signals of a different molecular weight appeared on the membrane. Taken together, these results show that when HOCl reacts with MPO the link between the heavy and light subunits is broken, the heme group is destroyed, and higher molecular weight aggregates are formed.

Next, we used size exclusion chromatography to confirm HOCl converts MPO to high molecular weight polymers. For the MPO/H_2O_2/Cl^- system with a 400-fold excess of hydrogen peroxide over protein, MPO was partially converted to two small early eluting peaks suggesting initial formation of dimers and trimers (Fig. 3D). With increasing concentrations of hydrogen peroxide, more of the native protein was lost, and most of the modified protein eluted as a broad peak around 12 min, suggested the formation of high molecular weight polymers. Molecular weight standards were used to create a standard curve to calculate the size of the polymers formed and to extrapolate for polymers which eluted close to the void volume of the column. The number of MPO molecules per polymer was estimated to be between 15 (at 500-fold excess of hydrogen peroxide to protein) and 18 (at 1500-fold excess of hydrogen peroxide to protein). When MPO was treated with reagent HOCl, it gave similar high molecular weight species (Fig. S2C). The major difference to the hydrogen peroxide/chloride system was the strong broadening of the peaks which suggested the presence of a highly mixed population of polymers, formed by three to 18 MPO molecules. Dynamic light scattering (DLS) was used to determine the radii of the polymers formed in the two different oxidant systems (Fig. 3E and Fig. S2D). The predominant sizes determined by DLS corresponded well with the size exclusion chromatography results, suggesting that the reaction of MPO with hydrogen peroxide in the presence of chloride generates distinct high molecular weight polymers of approximately 17 MPO monomers from an oxidant to protein ratio of 500:1 (Fig. 3E). For treatment with reagent HOCl, the DLS data suggests less defined polymer formation as also seen in the size exclusion chromatography.

To study the effect of oxidative modifications on protein stability, we measured thermal unfolding with electronic circular dichroism at 222 nm after treatment of MPO with HOCl. As known from previous studies, MPO is a highly stable protein with a very high thermal stability midpoint transition of 80.9 °C [41]. We confirmed that MPO is very stable, with or without high chloride concentration, as shown by the thermodynamic data summarized in Table 1. However, exposure of MPO to HOCl reduces its thermal stability and protein integrity. Treatment of MPO with a 200-fold excess of HOCl resulted in a dramatically reduced melting temperature, which was also reflected by significant changes in the enthalpic and entropic contributions to the free enthalpy of unfolding (Table 1).

Mass spectrometric analysis of oxidatively modified MPO was used to obtain molecular details of which amino acids residues are modified when MPO reacts with HOCl. In this experiment MPO was treated with 7.5 consecutive additions of 300 μM hydrogen peroxide in the presence of 140 mM chloride resulting in a final oxidant to protein ratio of 1500. The oxidized protein was digested with trypsin and the resulting tryptic peptides were analysed by LC/MS/MS using an Orbitrap mass spectrometer. The oxidative modifications detected are depicted on the full amino acid sequence of MPO in Fig. 4A and shown on a model of its 3D structure in Fig. 4B. A full list of the modifications is given in Table S1. The amino acid sequence coverage was 86%. As expected, oxidation of methionine residues to methionine sulfoxide and methionine sulfone was most common. The two disulfide bridges (Cys1-Cys14 and Cys119-Cys143) were oxidized and resulted in the formation of cysteine sulfonic acid on the residues Cys14 and Cys143. Cys1 and Cys153 were on peptides which were not detected by LC-MS/MS. Cys153 is the cysteine residue which forms the disulfide bridge between two MPO monomers, resulting in the homodimer of mature MPO. Our analysis also revealed the chlorination of three tyrosine residues to produce 3-chlorotyrosines. No other major modifications were identified. This finding does not exclude the possibility that other modifications were present but not detected. Table S2 summarizes the solvent accessibility of the identified modified amino acid residues as well as their distance to the heme iron. Oxidized amino acids are distributed evenly throughout the protein and are not only localized near the catalytic site. Many of the oxidized amino acid residues were not surface exposed, and therefore not directly oxidized by HOCl, but rather by an oxidized amino acid residue intermediate or chloramines.

3.3. Oxidative inactivation of myeloperoxidase when neutrophils phagocytose bacteria

Previously, it was found that when neutrophils ingested *Staphyloccocus aureus* at a ratio of 1000 bacteria per neutrophil, they lost approximately half of their MPO activity. Up to eight percent of the MPO activity was released into the extracellular medium [21]. Following our

Table 1
Thermodynamic data of thermal unfolding (calculated from van 't Hoff plot) for MPO with and without 200 fold excess of HOCl in the presence of 467 mM NaCl followed by electronic dichroism at 222 nm. Melting temperature (T_m), enthalpic (ΔH_m) and entropic (ΔS_m) contribution to free enthalpy ($\Delta G_{25°C}$).

	MPO 467 mM NaCl ([θ]222)	MPO ([θ]222)	MPO 200 equ HOCl (1) ([θ] 222)	MPO 200 equ HOCl (2) ([θ] 222)
T_m (°C)	81.4 ± 0.1	79.7 ± 0.1	43.0 ± 0.2	64.5 ± 1.8
ΔH_m (kJ mol^{-1})	615 ± 35	532 ± 27	284 ± 18	300 ± 5
ΔS_m (J mol^{-1}K^{-1})	1733 ± 101	1507 ± 76	897 ± 58	882 ± 15
$\Delta G_{25°C}$ (kJ mol^{-1})	42.4	34.6	6.2	14.9

(A)

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CPEQDKYRTI TGMCNNRRSP TLGASNRAFV RWLPAEYEDG FSLPYGWTPG VKRNGFPVAL     60
ARAVSNEIVR FPTDQLTPDQ ERSLMFMQWG QLLQHDLDFT PEPAARASFV TGVNCETSCV    120
                                  #    *
QQPPCFPLKI PPNDPRIKNQ ADCIPFFRSC PACPGSNITI RNQINALTSF VDASMVYGSE    180
          *                                  *
EPLARNLRNM SNQLGLLAVN QRFQDNGRAL LPFDNLHDDP CLLTNRSARI PCFLAGDTRS    240
SEMPELTSMH TLLLREHNRL ATELKSLNPR WDGERLYQEA RKIVGAMVQI ITYRDYLPLV    300
                  *
LGPTAMRKYL PTYRSYNDSV DPRIANVFTN AFRYGHTLIQ PFMFRLDNRY QPMEPNPRVP    360
LSRVFFASWR VVLEGGIDPI LRGLMATPAK LNRQNQIAVD EIRERLFEQV MRIGLDLPAL    420
NMQRSRDHGL PGYNAWRRFC GLPQPETVGQ LGTVLRNLKL ARKLMEQYGT PNNIDIWMGG    480
VSEPLKRKGR VGPLLACIIG TQFRKLRDGD RFWWENEGVF SMQQRQALAQ ISLPRIICDN    540
                  *
TGITTVSKNN IFMSNSYPRD FVNCSTLPAL NLASWREAS                           579
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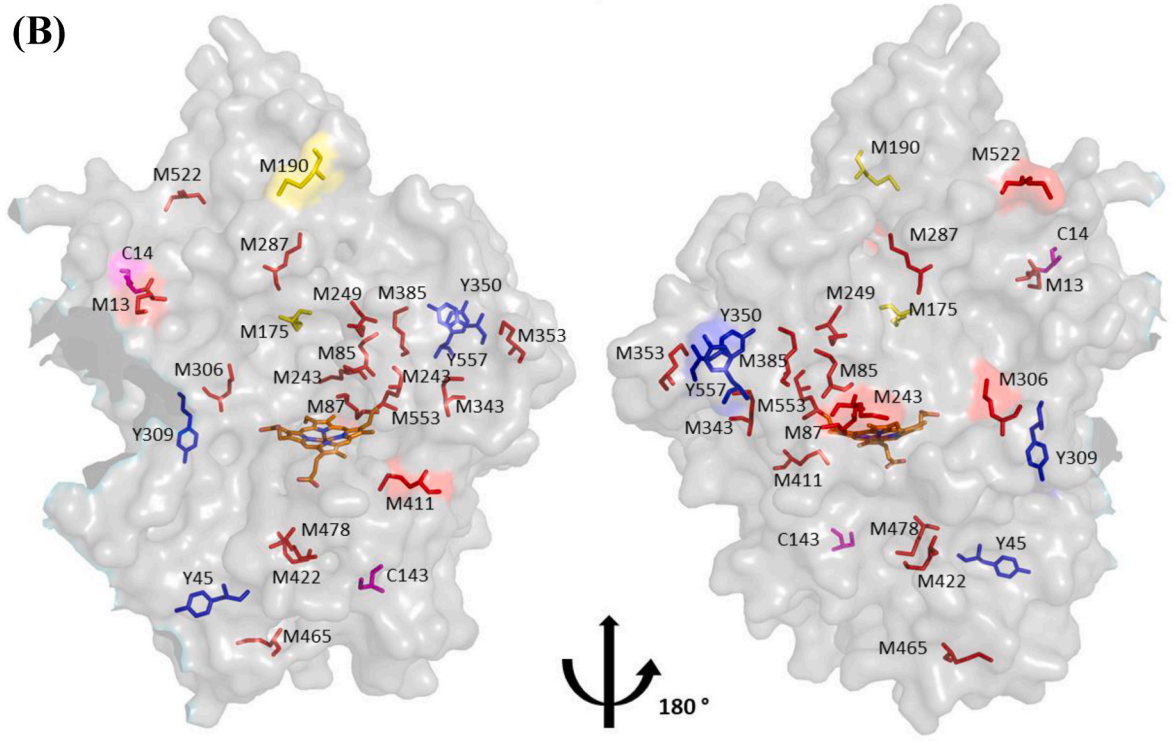

Fig. 4. Oxidative modifications of MPO detected by LC-MS/MS/Orbitrap analysis. MPO (1.5 μM) was reacted with 7.5 bolus additions of 300 μM hydrogen peroxide in the presence of 140 mM chloride, digested with trypsin, and the resulting peptides analysed by LC/MS/MS on an Orbitrap mass spectrometer. (**A**) The amino acid sequence of the MPO light chain (106 amino acids) is shown in blue, while that for the heavy chain (467 amino acids) is shown in black. The small excised hexa-peptide between the light and heavy subunit is depicted in grey. Important catalytic residues are highlighted in yellow, and amino acid residues involved in the covalent link with the prosthetic group are in green. N-glycosylation sites are marked by *, the cysteine that forms the disulfide bridge between the subunits is marked by # and ligands of the calcium ion binding site are underlined. The amino acid numbering for MPO is based on the mature protein with the first cysteine in the small polypeptide designated as residue 1. The amino acid sequence coverage by LC-MS/MS (86%) is depicted in bold letters, oxidized methionine residues (sulfoxide) are highlighted in red, and dioxidized methionine residues (methionine sulfone) are highlighted in red and underlined. Cysteine sulfonic acid residues are highlighted in pink. 3-chlorotyrosines identified on oxidized MPO are highlighted in turquoise and the 3-chlorotyrosine detected in neutrophil supernatant is depicted in doubly underlined turquoise. **(B)** The 3D structure of MPO illustrating the modified amino acid residues was created with Pymol using data from PDB 3f9p in the Protein Data Bank. The heme prosthetic group is shown in orange. Oxidized methionine residues are depicted in red, unoxidized methionines are highlighted in yellow. Cysteine sulfonic acid residues are illustrated in pink, whereas chlorinated tyrosines are shown in blue. This code is also used to show solvent exposed amino acid residues, which are depicted as coloured surfaces. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

analysis of isolated granule extract MPO and purified MPO systems, we sought to examine enzyme inactivation during phagocytosis of bacteria. We used an inoculum of 100 bacteria per neutrophil. After ingestion of bacteria, neutrophils were pelleted, lysed and MPO activity was measured in lysates and the original supernatant. Initially, we used the TMB assay to measure MPO activity as in Fig. S1A, but the assay was prone to interference from constituents of the cell extracts. Therefore, we extracted MPO from cell lysates and supernatants using the cation

exchanger CM-sepharose before applying samples to the TMB assay (Fig. S3A). Overall the results agreed with the earlier study and showed that MPO activity declined after phagocytosis [21]. Inactivation was oxidant-dependent because it was prevented by diphenyliodonium (DPI), which inactivates the neutrophil's NADPH-oxidase [43]. The oxidative effect was most pronounced with extracellular MPO.

However, there were limitations with measuring only MPO activity because it does not report on the amount of MPO protein and we were concerned that CM-sepharose may not capture oxidized MPO protein. Therefore, in line with the experiments on the granule extract and purified MPO, we used the MPO ELISA (as in Fig. 1A–C) to measure MPO activity and MPO protein that was released by activated neutrophils without prior purification using CM-sepharose. After 30 min incubation with bacteria, there was a several-fold increase (6 ± 3-fold, across 7 experiments) in the amount of extracellular MPO (protein) released compared to non-phagocytic neutrophils (Fig. 5A). There was no effect of DPI or methionine on the amount of MPO released. However, with DPI present, higher MPO activity was recovered in the supernatants from phagocytic neutrophils ($p < 0.03$). In contrast to DPI, methionine did not affect MPO activity (Fig. 5A). The specific activity of MPO, calculated as the ratio of MPO activity to MPO protein, was significantly decreased by 60% in the supernatants compared to that in untreated neutrophils (Fig. 5B). As reported in Fig. 1., oxidized MPO not detected by the ELISA would have been inactivated to a larger degree. DPI restored the specific activity ($p = 0.0001$), which indicates MPO had been oxidatively inactivated. The inability of methionine to protect against the loss of activity, suggests that oxidative inactivation occurred before the MPO was released to the extracellular fluid.

To determine whether MPO released from neutrophils had been inactivated by HOCl, we aimed to identify a modification specific to HOCl that was present in the inactive protein but not in the active enzyme. Although methionine oxidation was the major modification in MPO inactivated by HOCl (Fig. 4), formation of methionine sulfoxide is not specific to this oxidant. Therefore, we focused our attention on chlorination of tyrosine residues because even though they were not as abundant as methionine modifications, they are specific to HOCl. Purified MPO was treated with hydrogen peroxide (oxidant to protein ratio of 150) in the presence of chloride and digested with trypsin. The *m/z* values of MPO tryptic peptides containing 3-chlorotyrosine modifications, including the expected 3:1 isotope ratio for a chlorine containing peptide, were predicted and searched for by LC/MS using a triple quadrupole mass spectrometer. A 3-chlorotyrosine modification to the N-terminal tyrosine of the MPO tryptic peptide YLPTYR was detected and characterised (shown in Fig. 4 as residues 309–314 with the fragmentation patterns in Figs. S4A–C). A multiple-reaction monitoring (MRM) method was set up to detect the peptides containing the two chlorine isotopes ^{35}Cl (Y(^{35}Cl)LPTYR) and ^{37}Cl (Y(^{37}Cl)LPTYR), along with the parent peptide (YLPTYR). A control tryptic peptide (VVLEGGIDPILR) from MPO was also monitored to confirm the overall amount of MPO in the supernatants (Table S3). When MPO was treated with hydrogen peroxide in the presence of chloride, digested and analysed by LC/MS/MS, peaks of the ^{35}Cl and ^{37}Cl isotopes of Y(Cl)LPTYR coeluted and were detected at an approximate 3:1 ratio as expected for a monochlorinated peptide (Fig. 6A).

Neutrophils were then stimulated with opsonised bacteria under serum-deprived conditions to prevent other abundant proteins from interfering with the detection of MPO tryptic peptides. Under these conditions, MPO released from the neutrophils was oxidatively inactivated in accord with the results presented above (compare Fig. 6C with Fig. 5B). When proteins in the supernatants were digested with trypsin and analysed by LC/MS/MS, both peaks for the isotopes of chlorinated YLPTYR were present and at the expected ratio (Fig. 6B). The proportion of Y(^{35}Cl)LPTYR produced relative to its parent peptide, YLPTYR, was significantly increased when bacteria were added to neutrophils, and this increase was abrogated in the presence of DPI (Fig. 6D). Measurement of the control MPO peptide, VVLEGGIDPILR, confirmed the presence of MPO in the supernatants and showed that the amount of MPO released from neutrophils was significantly increased after phagocytosis of bacteria. However, in contrast to the chlorinated peptide, addition of DPI caused only a small decrease in the MPO control peptide (Fig. 6E). Collectively, these results demonstrate that when neutrophils phagocytosed bacteria, they released MPO that was partially inactivated and had reacted with HOCl.

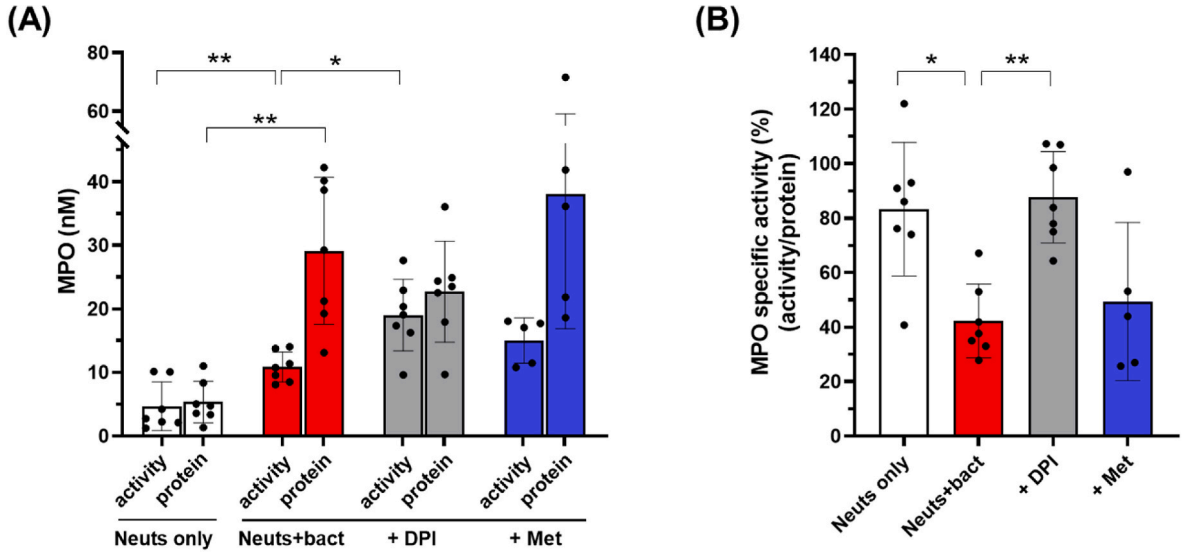

Fig. 5. Loss of activity in neutrophil-released MPO after phagocytic stimulation of the NADPH oxidase. (A) Neutrophils (10^7/mL) were incubated with serum-opsonised *S. aureus* (2×10^8/mL) at 37 °C with end-over-end mixing for 30 min. Incubations were in HBSS containing 10% serum, in the absence or presence of 5 μM DPI (n = 7) or 1 mM methionine (n = 5). Neutrophils were then pelleted by centrifugation at 100*g* and the supernatant was analysed by MPO ELISA. The supernatant was also collected from neutrophils incubated at 37 °C without bacteria or mixing (Neuts only). (**B**) The specific activity of MPO was calculated from the ratio of MPO activity to MPO protein as determined in (A). Significant differences between treatments, using paired data, were identified with Šidák's mixed effects multiple comparisons test (for A; *$p < 0.03$, **$p < 0.003$, and B; *$p < 0.04$, **$p = 0.0001$). Datapoints in all of the graphs are from seven separate experiments using six different donors, with inter-experiment means ± SD.

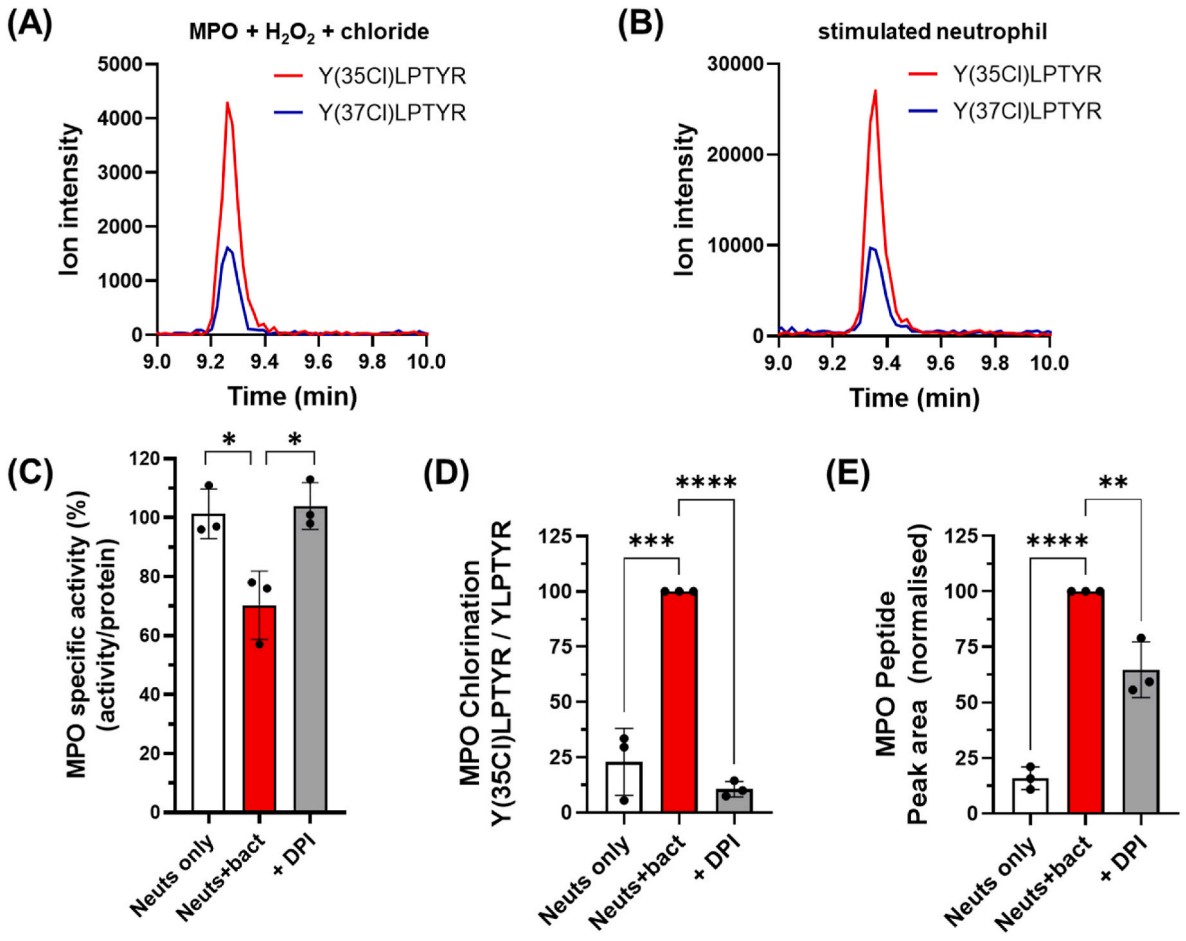

Fig. 6. MPO released from phagocytosing neutrophils contains a chlorinated tryptic peptide. (A) Purified MPO treated with 225 μM hydrogen peroxide in the presence of 140 mM chloride (~80 ng MPO), and **(B)** supernatants (~14 μg protein) from neutrophils that had phagocytosed serum-opsonised *S. aureus* in the presence and absence of DPI as described for Fig. 5 (except without 10% serum during phagocytosis) were analysed for the two isotopes of the tryptic peptide Y(Cl)LPTYR using the LC-MS/MS QTrap (representative of three separate experiments) and **(C)** for the specific activity of MPO by ELISA, *$p < 0.05$ by one-way ANOVA and Dunnett's multiple comparison. **(D)** Y(^{35}Cl)LPTYR/YLPTYR peptide peak area ratio (mean ± SD, n = 3) and **(E)** the non-oxidizable MPO tryptic peptide VVLEGGIDPILR peak area in neutrophil supernatant samples (mean ± SD, n = 3) were determined for neutrophils alone, and those phagocytosing bacteria with or without the NADPH-oxidase inhibitor DPI. Peptides were quantified by fragmenting the doubly-charged parent ion and monitoring an abundant characteristic fragment ion (Table S1 & Fig. S1). ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$ by one-way ANOVA analysis followed by Tukey's multiple comparisons.

4. Discussion

MPO is well known to inactivate itself through the generation of its major product HOCl [19,20,22,24]. However, whether this self-inactivation occurs during phagocytosis and affects the antimicrobial function of MPO has received comparatively little attention [21,44]. Here we found that when neutrophils phagocytosed bacteria they released MPO, which retained only 40% of its activity. Also, the MPO released from neutrophils contained a chlorinated tyrosine residue. Both the decline in activity and chlorination of the protein were dependent on an active NADPH-oxidase. Based on these findings, we propose that HOCl generated during phagocytosis reacts with MPO within phagosomes and contributes to its partial inactivation. We found that MPO is able to absorb a high oxidative insult through oxidation of methionine residues and formation of chloramines and dichloramines on amine residues. These modifications occurred before HOCl destroyed the enzyme's heme groups, and MPO lost activity, fragmented, and formed high molecular weight aggregates. Consequently, MPO is likely to sustain HOCl production during phagocytosis even though it will, along with other neutrophil proteins, be a target for oxidation.

In agreement with previous investigations [19,20], we found that when hydrogen peroxide was added to a neutrophil granule extract containing chloride and MPO, HOCl was generated and eventually inactivated the enzyme. MPO is not the only protein that is oxidized by HOCl when hydrogen peroxide is added to neutrophil granule extracts. As already shown [19] lysozyme is the neutrophil granule enzyme most susceptible to HOCl followed by MPO, β-glucuronidase, collagenase, and gelatinase. Elastase is extremely resistant to oxidative inactivation. Here, we have identified lactoferrin as another target prone to modification by HOCl. Together, these results demonstrate that even in the presence of multiple other proteins, as occurs within neutrophil phagosomes, MPO is susceptible to its own oxidant.

By using a dual ELISA for activity and protein, we were able to demonstrate that not only does MPO progressively lose activity as it generates HOCl, but that it also undergoes protein modifications that prevent it from being recognised in the ELISA. Whether the unrecognised MPO retained or lost activity could not be established. These protein modifications were not apparent on SDS-PAGE gels or western blots, which demonstrates that MPO is susceptible to subtle alterations of its amino acid resides as opposed to the fragmentation and aggregation that was readily observed at higher doses of hydrogen peroxide. The dominant protein modifications to MPO that were identified by Orbitrap mass spectrometry included methionine oxidation and some formation of 3-chlorotyrosine. Chloramines would not be detected by mass spectrometry as they are likely to be converted back to amines when the protein was reduced and alkylated for tryptic digestion. Minor

modifications would also not be detected by this mass spectrometry technique as it selected for the most abundant tryptic peptides. Low levels of methionine oxidation and chloramine formation could be tolerated by the enzyme but more extensive formation of methionine sulfoxide and chloramines would affect the charge state of the protein, which may have been responsible for its aggregation as we found previously for myoglobin [45]. Fragmentation of the cross-link between the heavy and light subunit may result from the reaction of HOCl with the heme, which both subunits are linked to Ref. [6].

To gauge how much HOCl MPO can tolerate before it inactivates itself, we studied the oxidative insult to the purified enzyme. Purified MPO appeared much more resistant to the HOCl it generated from hydrogen peroxide than reagent HOCl. This difference most likely arises from the competing reaction of HOCl with hydrogen peroxide ($k = 1.5 \times 10^3\ M^{-1}\ s^{-1}$ at pH 7.4) [46]. In the presence of chloride, the enzyme was approximately 50% inactivated after absorbing 200 molecules of reagent HOCl per monomer of protein, suggesting that the enzyme can undergo substantial oxidative modifications before it loses activity. Interestingly, at low doses of reagent HOCl, chloride protected the enzyme from inactivation. This protection is best explained by chloride reacting with Compound I. This reaction would prevent the spontaneous decay of Compound I to a redox intermediate (Compound I*) that damages the heme prosthetic group (reactions 1–3) [23]. It is presumed that like other peroxidases this decay of Compound I involves transfer of the π-cation radical on the heme to a protein residue, such as tyrosine or tryptophan [47]. Chloride would essentially prevent damage of the heme groups by generating HOCl which in turn reacts with amino acids residues. As expected, based on previous investigations into the kinetics of HOCl reacting with the heme groups of MPO, the inactivation reaction was fast [26,27]. Under physiological conditions other (pseudo)halides or peroxidase substrates may be present in the neutrophil phagosome. However, they are likely to be present at relatively low concentrations compared to chloride and superoxide. Consequently, they are unlikely to have a major impact on the activity of MPO. The one exeption is thiocyanate which may have a protective effect on MPO activity, either by competing with chloride or by scavenging HOCl.

$$HOCl + MPO^{3+} \rightarrow \text{Compound I} + Cl^- \quad (1)$$

$$\text{Compound I} + Cl^- \rightarrow MPO^{3+} + HOCl \quad (2)$$

$$\text{Compound I} \rightarrow \text{Compound I}^* \quad (3)$$

We contend that the partially inactive and chlorinated MPO detected outside phagocytosing neutrophils originated from within phagosomes. In support of this proposal, early work from our laboratory showed that when neutrophils phagocytose *E coli*, bacterial iron was released inside phagosomes, most of which became bound to lactoferrin and was eventually found extracellularly still bound to lactoferrin [48]. Thus, it is apparent that during phagocytosis neutrophils release their own proteins into the extracellular environment. A similar conclusion was reached by other groups who detected more extracellular peroxidase activity from phagocytosing than resting neutrophils [21,49]. However, in these earlier studies the authors did not measure total MPO protein so they would have underestimated the amount of MPO released from neutrophils. In addition, we have previously shown that as a consequence of phagocytosis, neutrophil proteins inside phagosomes are more highly chlorinated than either their cytosolic proteins or those from ingested bacteria [10,14]. Given that the inactivation and chlorination of the extracellular MPO was not affected by methionine, which scavenges HOCl, we conclude that the oxidative modifications of MPO occurred inside neutrophils before the protein was released to the outside of neutrophils.

Previous kinetic modelling of the activity of MPO within neutrophil phagosomes suggests that a 60% loss in activity of the enzyme will not affect HOCl production appreciably [5]. This is because the concentration of MPO within phagosomes is estimated to be up to a millimolar, sufficiently high to ensure that the supply of superoxide by the NADPH-oxidase is the rate limiting step in HOCl production. One caveat to this conclusion is that the degree of inactivation of MPO we detected extracellularly may underestimate the degree to which the enzyme is inactivated inside phagosomes. However, based on modelling, a 90% loss in MPO activity would still allow oxidant production to be maintained at 50% of the maximum rate [5].

Other neutrophils proteins are chlorinated in the phagosome [14]. Their fate will be determined by their susceptibility to HOCl [19]. Just as with MPO, their antimicrobial or digestive roles may not necessarily be fully compromised. For example, as described above, lactoferrin still maintains at least some of its ability to chelate bacterial iron during phagocytosis even though we found here that it is prone to oxidation by HOCl [48]. Elastase would be expected to be fully functional while lysozyme is the neutrophil granule enzyme that is most likely to lose its activity.

In conclusion, we have found that MPO inactivates itself during phagocytosis but sufficient activity should be retained to maintain HOCl production. The HOCl-dependent protein modifications that occur during phagocytosis may simply reflect collateral damage as neutrophils try to kill ingested bacteria with oxidants. Alternatively, these modifications may restrict oxidative and proteolytic activity to what is required within phagosomes [19], aid in the digestion of phagosomal proteins [50], or they may even contribute to the demise of ingested bacteria through subsequent reactions of chloramines [51]. Future studies should be directed at determining whether MPO or other specific neutrophil proteins are preferentially oxidized or chlorinated, and whether these modifications aid or hinder innate immunity.

Author contributions

Martina Paumann-Page contributed to conceptualization, funding acquisition, design and performance of experiments and methodology, data curation and analysis, preparation and writing of manuscript, statistical analyses, discussion, coordination. Review, editing and submission. Her experimental work led to Fig. 1F, 2A-F, 3A-F, 4A, S2A-D and Suppl. Table 1.

Louisa V. Ashby performed experiments with neutrophils stimulated by bacteria and subsequent activity and protein measurements, development of methodology thereof, contributed to discussion and manuscript preparation, editing. Her work is depicted in Fig. 5A–B and Fig. S3A-C.

Irada Khalilova performed experiments with granule extracts leading to Fig. 1A–E and S1A-B. Current address: Centre for Cell Pathology Research, Khazar University, Azerbaijan.

Nicolas Magon performed LS-MS/MS/QTrap analysis, development of methodology, leading to Fig. 6A–D, S4A-D and Suppl. Table 3.

Stefan Hofbauer performed and assisted with biophysical measurements leading to Fig. 3E, S2D and Table 1 and generated Fig. 4B to illustrate oxidative modifications.

Louise N. Paton assisted with mass spectrometric expertise.

Christian Obinger, Paul G. Furtmüller and **Anthony J. Kettle** contributed to conceptualization, funding acquisition, design, discussions and writing and editing of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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LC/MS/MS/Orbitrap mass spectrometry was performed by Dr Torsten Kleffmann, Centre for Protein Research, University of Otago, Dunedin, New Zealand, email cpr@otago.ac.nz.

The authors declare to have no competing or conflicting interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rbc.2023.100008.

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